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**Abstract**

A heterologous competitive indirect enzyme-linked immunosorbent assay (ciELISA) for the determination of the furaltadone metabolite 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) was developed. AMOZ was derivatised with 2-(4-formylphenoxy)acetic acid or 2-(3-formylphenoxy)acetic acid to obtain two novel immunizing haptens. The ability of these haptens in producing specific polyclonal antibodies against the nitrophenyl derivative of AMOZ (NPAMOZ) was compared with that of traditional immunizing haptens (derivatised AMOZ with 3-carboxybenzaldehyde or 4-carboxybenzaldehyde). The results indicated that the novel immunizing haptens were able to produce antibodies with almost a two-fold improvement in sensitivity of the ciELISA for NPAMOZ in comparison with the existing antibody based ELISAs. The differences in sensitivity were explained by the molecular modeling of the lowest energy conformations of NPAMOZ and the haptens. Another novel hapten, derivatised AMOZ with 2-oxoacetic acid, was synthesized and used as a heterologous coating hapten. The results showed that this strategy of using only a partial structure of the target molecule as the coating hapten was able to obtain a two to three-fold improvement in sensitivity. This study provided a modern approach for the development of an immunoassay with improved sensitivity for the metabolites of nitrofurantoin antibiotics.

**Keywords:** Furaltadone; 3-amino-5-morpholinomethyl-2-oxazolidinone; haptens; enzyme-linked immunosorbent assay; molecular modeling.

## 1. Introduction

Nitrofuran antibiotics including furazolidone, furaltadone, nitrofurazone and nitrofurantoin are a class of synthetic broad spectrum antibiotics which have been widely used both prophylactically and therapeutically for various bacterial and protozoan infections in fish, prawn, bees, swine, cattle and poultry [1]. However, since the early 1990s, various experimental animal studies have shown that nitrofurans and their metabolites have potential mutagenicity and genotoxicity [2]. As a consequence, nitrofurans have been banned for use in animal husbandry in the European Union (EU) and other countries [2]. The EU has set the minimum required performance limits (MRPLs) to be  $1 \mu\text{g kg}^{-1}$  for each nitrofuran metabolite in food of animal origin [3]. Nonetheless, the illegal use of nitrofuran still exists in many countries, particularly developing countries, mainly due to their effectiveness and low cost. Therefore, monitoring programs are deemed necessary to prevent the consumption of potentially harmful nitrofuran antibiotic residues in food.

Nitrofuran antibiotics are rapidly metabolized *in vivo* and do not persist as residues of the parent drugs in edible tissues [4]. Their metabolites can bind to tissue proteins and may persist for considerable periods of time in animal tissues. Therefore, the analytical detection of furaltadone, furazolidone, nitrofurazone and nitrofurantoin can be monitored through the measurement of their metabolites, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD), respectively [5]. Instrumental analytical methods such as liquid chromatography with ultraviolet (UV) or UV photodiode array detection and the coupling of high performance liquid chromatography and liquid chromatography electro-spray ionisation to tandem mass

spectrometry (HPLC-MS/MS and LC-ESI MS/MS, respectively) are well established for the determination of nitrofurantoin antibiotics and their metabolites. A detailed review on the residual analysis of nitrofurans and their metabolites was recently published by Vass et al. [2]. The instrumental methods can offer high sensitivity and specificity and have the potential for simultaneous determination of several nitrofurans, but the associated high-costs, time-consuming labour requirements and practicalities of the instrumentation have inhibited broadening the scope of monitoring particularly to field-screening scenarios. As an alternative, immunoassays based on the interaction of antibody and antigen provide a low cost, portable and high throughput screening method capable of the sensitive determination of nitrofurantoin metabolites. The first enzyme-linked immunosorbent assay (ELISA) capable of detecting the furazolidone metabolite (AOZ) was developed by Cooper et al. [6] under the support of a multi-national EU research project "FoodBRAND" [2]. Since then, immunoassays for the determination of AOZ [7-9], SEM [10-12], AHD [13, 14] and AMOZ [15] were reported in succession.

As the nitrofurantoin metabolites are relatively small molecular weight compounds, there always exists great difficulty in producing antibodies directly which are specific to the metabolites. As nitrofurantoin metabolites are covalently bound to proteins and are released from the tissues under slightly acidic conditions, they are derivatised with *o*-nitrobenzaldehyde (*o*-NBA) to form nitrophenyl (NP) derivatives prior to detection [16]. Therefore, immunoassays for nitrofurantoin metabolites are developed based on antibodies specific to their NP derivatives (e.g. NPAOZ, NASEM, NPAHD and NPAMOZ) [6-15]. In most studies, nitrofurantoin metabolites were derivatised with 3-carboxybenzaldehyde (3-CBA) or 4-

carboxybenzaldehyde (4-CBA) to form immunizing haptens [6, 7, 9-15]. The haptens were coupled to carrier proteins through the carboxylic acid spacer to generate immunogens. However, in relation to hapten design for small molecular weight compounds, a suitable length of spacer between the hapten and the carrier protein should be beneficial for producing desired antibodies [17]. A previous study indicated that the conformation of a hapten could be altered when too short a spacer is employed for its conjugation to the carrier protein [18].

Therefore, a question posed in this study was to determine whether it was possible to improve the assay sensitivity for nitrofurantoin metabolites by introducing a certain length of spacer between the phenyl ring and the carrier protein using the furaltadone metabolite AMOZ as the metabolite model. The first objective was to form two novel immunizing haptens, which contained a methoxyacetic acid spacer (Fig. 1) by derivatising AMOZ using 2-(4-formylphenoxy)acetic acid or 2-(3-formylphenoxy)acetic acid. Secondly, the ability of these novel haptens for producing specific antibodies against NPAMOZ was to be evaluated and compared with the traditional immunizing haptens, derivatised AMOZ with 3-CBA and 4-CBA (Fig. 1). Molecular modeling will be applied to obtain the lowest energy conformation of the target molecule and haptens to elucidate the results of ciELISA when each of the different antibodies raised to the various haptens are utilized. A further aim was to evaluate the effect of different combinations of heterologous assay format on assay sensitivity using each of the synthesized haptens and an additional novel hapten synthesized by derivatising AMOZ with 2-oxoacetic acid which provides a short spacer between the hapten and protein.

Finally, the ELISA method for determination of AMOZ was developed and applied to fish and shrimp samples, and the results were validated by standard LC-MS/MS method.

## 2. Experimental

### 2.1. Materials and reagents

Furaltadone, AMOZ, NPAMOZ, 4-CBA, 3-CBA, 2-(4-formylphenoxy)acetic acid, 2-(3-formylphenoxy)acetic acid, 2-oxoacetic acid, *o*-NBA, bovine serum albumin (BSA), ovalbumin (OVA), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), 3,3',5,5'-tetramethylbenzidine (TMB), complete and incomplete Freund's adjuvants were purchased from Sigma-Aldrich (Shanghai, China). *N,N*-dimethylformamide (DMF), dimethyl sulphoxide (DMSO), Tween-20, methanol and hexane were obtained from Damao Chemical Reagent Co., Ltd. (Tianjin, China). Peroxidase-conjugated goat anti-rabbit IgG (IgG-HRP) was obtained from Boster Biotech Co., Ltd (Wuhan, China). Thin-layer chromatography (TLC) was performed on 2.5 mm pre-coated silica gel F254 (200 mesh, Qingdao Haiyang Chemical Co., Ltd., China) on glass sheets. Polystyrene ELISA plates were obtained from Jiete Biotech Co., Ltd (Guangzhou, China). All other chemicals and organic solvents were of analytical grades.

### 2.2. Instrumentation

Ultraviolet-visible (UV-Vis) spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan). ESI-MS analysis for haptens was performed using an



Agilent HP1100 series (Agilent, Palo Alto, CA). Nuclear magnetic resonance (NMR) spectra were obtained with both the DRX-400 and DRX-600 NMR spectrometers (Bruker, Germany-Switzerland). LC-MS/MS analysis for AMOZ was carried out by using the 1200 series LC system (Agilent Technologies, USA) equipped with the Agilent 6410 Triple Quad LC-MS System (Agilent Technologies, USA). ELISA plates were washed using Multiskan MK2 microplate washer (Thermo Labsystems, USA). Absorbance was measured at 450 nm of wavelength using Multiskan MK3 microplate reader (Thermo Labsystems, USA).

### 2.3. Buffers and solutions

The buffers used in this study were as follows: 0.1 mol L<sup>-1</sup> phosphate buffer saline (PBS, pH 7.4) containing 2 mol L<sup>-1</sup> NaOH for sample neutralization; 50 mmol L<sup>-1</sup> carbonate buffer (pH 9.6) for ELISA coating, 10 mmol L<sup>-1</sup> PBST solution (10 mmol L<sup>-1</sup> PBS containing 0.05% Tween-20, pH 7.4) for washing, 0.1 mol L<sup>-1</sup> citrate and sodium phosphate buffer (pH 5.4) for substrate buffer and 2 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> as the stopping reagent. TMB solution was prepared by addition of 10mL substrate buffer and 150 µL of 15 µg L<sup>-1</sup> TMB-DMF and 2.5 µL of 6% (w/v) H<sub>2</sub>O<sub>2</sub>.

### 2.4. Synthesis and characterization of haptens

Five haptens (Fig. 1) were designed and synthesized. In brief, 1.0 mmol of AMOZ in 5 mL methanol was added to a stirring solution of 1.5 mmol of benzaldehyde derivative (4-CBA, 3-CBA, 2-(4-formylphenoxy)acetic acid and 2-(3-formylphenoxy)acetic acid, respectively) or 2-oxoacetic acid in 10 mL methanol. The mixture was left standing for 3 h at room temperature. Thin-layer chromatography of the reaction mixture demonstrated formation of

the target product (elution in 10% methanol in chloroform). The mixture was filtered and washed with ethanol several times to remove unreacted reagents. After removal of methanol by evaporation, the synthesized haptens were confirmed by ESI-MS and NMR.

Hapten 1 (4-((5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino)methyl)benzoic acid): The yield was 68%. MS (ESI negative)  $m/z$ :  $[(M-H)^-]$ .  $^1H$ -NMR (600MHz,  $d_5$ - Pyridine, TMS):  $\delta$  8.45 (d,  $J=8.27$  Hz, 2H); 8.01 (d,  $J=8.31$  Hz, 2H); 7.95 (s, 1H); 4.97 (m, 1H); 4.11 (t,  $J=8.87$  Hz, 1H); 3.80 (dd,  $J=8.80$ , 6.71 Hz, 1H); 3.71-3.65 (m, 4H); 2.68 (dd,  $J=13.34$ , 6.23 Hz, 1H); 2.62 (dd,  $J=13.38$ , 5.74 Hz, 1H); 2.52-2.47 (m, 4H).  $^{13}C$ -NMR (150MHz,  $d_6$ -DMSO, TMS):  $\delta$  166.88, 153.07, 142.41, 138.27, 131.33, 129.69, 126.81, 70.93, 66.05, 60.86, 53.68, 45.77.

Hapten 2 (3-((5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino)methyl)benzoic acid): The yield was 69%. MS (ESI negative)  $m/z$ : 332  $[(M-H)^-]$ .  $^1H$ -NMR (400 MHz,  $d_5$ - Pyridine, TMS):  $\delta$  8.86 (s, 1H), 8.41 (d,  $J=7.71$  Hz, 1H), 8.13 (d,  $J=7.81$  Hz, 1H), 7.98 (s, 1H), 7.50 (t,  $J=7.71$  Hz, 1H), 4.96 (m, 1H), 4.11 (t,  $J=8.83$  Hz, 1H), 3.80 (dd,  $J=8.79$ , 6.73 Hz, 1H), 3.72-3.65 (m, 4H), 2.69 (dd,  $J=13.32$ , 6.13 Hz, 1H), 2.62 (dd,  $J=13.35$ , 5.82 Hz, 1H), 2.55-2.45 (m, 4H).  $^{13}C$ -NMR (150MHz,  $d_6$ -DMSO, TMS):  $\delta$  166.85, 153.17, 142.75, 134.70, 131.35, 131.00, 130.20, 129.10, 127.38, 70.92, 66.07, 60.88, 53.68, 45.77.

Hapten 3 (2-(4-((5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino)methyl)phenoxy)acetic acid): The yield was 72%. MS (ESI negative)  $m/z$ : 362  $[(M-H)^-]$ .  $^1H$ -NMR (400 MHz,  $d_6$ - DMSO, TMS):  $\delta$  7.79 (s, 1H), 7.66 (d,  $J = 8.82$  Hz, 2H), 6.99 (d,  $J = 8.80$  Hz, 2H), 4.89 (m, 1H), 4.74 (s, 2H), 4.04 (t,  $J=8.96$  Hz, 1H), 3.63 (dd,  $J=8.90$ , 6.96 Hz, 1H), 3.58 (t,  $J=4.54$  Hz, 4H), 2.68 (dd,  $J=11.67$ , 4.48 Hz, 1H), 2.64 (dd,  $J=11.69$ , 3.93 Hz, 1H), 2.50-2.45 (m, 4H).  $^{13}C$ -NMR (150MHz,

$d_6$ -DMSO, TMS):  $\delta$  169.87, 158.96, 153.21, 143.29, 128.33, 127.33, 114.70, 70.68, 66.03, 64.41, 60.93, 53.66, 45.75.

Hapten 4 (2-(3-((5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino)methyl)phenoxy)acetic acid): The yield was 70%. MS (ESI negative)  $m/z$ : 362 ( $[M-H]^-$ ).  $^1H$ -NMR (600 MHz,  $d_5$ -Pyridine, TMS):  $\delta$  7.89 (s, 1H), 7.77 (s, 1H), 7.53 (d,  $J=7.51$  Hz, 1H), 7.35 (t,  $J=8.00$  Hz, 1H), 7.23 (d,  $J=8.23$  Hz, 1H), 5.00 (s, 2H), 4.94-4.87 (m, 1H), 4.01 (t,  $J=8.63$  Hz, 1H), 3.71-3.69 (m, 1H), 3.67-3.65 (m, 4H), 2.65 (dd,  $J=13.35$ , 6.25 Hz, 1H), 2.58 (dd,  $J=13.25$ , 5.49 Hz, 1H), 2.51-2.45 (m, 4H).  $^{13}C$ -NMR (150MHz,  $d_6$ -DMSO, TMS):  $\delta$  169.91, 157.95, 153.11, 143.32, 135.65, 129.81, 119.85, 116.08, 112.18, 70.79, 66.04, 64.37, 60.88, 53.67, 45.78.

Hapten 5 (2-(5-(morpholinomethyl)-2-oxooxazolidin-3-ylimino)acetic acid): The yield was 70%. MS (ESI negative)  $m/z$ : 256( $[M-H]^-$ ).  $^1H$ -NMR(600 MHz,  $d_5$ - Pyridine,TMS):  $\delta$  7.55 (s, 1H), 4.99-4.97 (m, 1H), 4.06 (t,  $J=8.82$  Hz, 1H), 3.75 (t,  $J=7.82$  Hz, 1H), 3.69-3.64 (s, 4H), 2.67 (dd,  $J=13.46$ , 6.10 Hz, 1H), 2.62 (dd,  $J=13.45$ , 5.54 Hz, 1H), 2.51-2.46 (m, 4H).  $^{13}C$ -NMR (150MHz,  $d_6$ -DMSO, TMS):  $\delta$  164.15, 152.65, 134.46, 71.53, 66.05, 60.67, 53.66, 45.52.

## 2.5. Preparation of hapten-protein conjugates

Four haptens (Hapten1 to Hapten4) were coupled to BSA for immunogens and all five haptens were coupled to OVA for coating antigens. Hapten (10  $\mu$ mol), NHS (20  $\mu$ mol) and DCC (20  $\mu$ mol) were dissolved in 500  $\mu$ L of DMF. The mixture was stirred gently at 4  $^{\circ}$ C overnight, and then centrifuged at 2500 g for 10 min. The supernatant (about 400  $\mu$ L) was added dropwise to BSA (90 mg) or OVA (60 mg) in 9.5 mL PBS (pH7.4). The conjugation mixture was then stirred at 4  $^{\circ}$ C for 12 hours and then purified on Sephadex G-25, using

0.01 mol L<sup>-1</sup> of NaHCO<sub>3</sub> as the eluent. The eluted conjugates were dialyzed against PBS (pH 7.4) and then freeze-dried before storage at 4 °C. Full wavelength (200–500 nm) UV-Vis scanning was used to confirm the structures of the final conjugates and the ratios of haptens to carrier proteins were determined by trinitro-benzene-sulfonic acid (TNBS) method [19].

## 2.6. Production of polyclonal antibodies

Immunogens were dissolved in 0.01 mol L<sup>-1</sup> PBS (1.0 mL) and emulsified with 1.0 mL of Freund's complete adjuvant. New Zealand male rabbits (supplied by the Guangdong Medical Laboratory Animal Center) weighting 1.5–2.0 kg were immunized subcutaneously at multiple sites (12–15 sites) on the back. Two rabbits were immunized for each immunogen. The number designated as R215 and R216 for hapten 1-BSA, R217 and R218 for hapten 2-BSA, R219 and R220 for hapten 3-BSA and R221 and R222 for hapten 4-BSA, respectively. Three weeks after the initial injection, booster immunizations were given intraperitoneally with the same amount of immunogen emulsified with incomplete Freund's adjuvant. Booster immunizations were performed every three weeks. Rabbits were bled through an ear vein one week after each booster injection following the third immunization. The blood samples were left to coagulate for 1.0 h at room temperature and overnight at 4 °C, and then centrifuged at 10,000 g for 10 min. The antisera (supernatant) were carefully collected, divide into aliquots and stored at –20 °C until used.

## 2.7. Titration of antisera

The affinity of each antiserum was determined by non-competitive indirect ELISA. For the ELISA, each well of a microtitre plate was coated with 100 µL of the coating antigen in

carbonate buffer ( $1 \text{ mg L}^{-1}$ ) overnight at  $4^\circ\text{C}$ . The wells were washed 5 times with PBST solution, and then blocked with 5% glycine and 5% sucrose in PBS buffer ( $200 \mu\text{L well}^{-1}$ ) for 3 h at  $37^\circ\text{C}$ . After washing 5 times with PBST solution, the wells were incubated with  $100 \mu\text{L}$  of diluted antiserum in PBST for 1 h at  $37^\circ\text{C}$  and washed 5 times with PBST solution. IgG-HRP diluted at 1: 10,000 in PBST was added ( $100 \mu\text{L well}^{-1}$ ). After incubation for 1 h at  $37^\circ\text{C}$  and washing 5 times with PBST solution, TMB solution was added to the wells ( $100 \mu\text{L}$ ) and incubated for 15 min. The reaction was stopped by addition of  $2 \text{ mol L}^{-1} \text{H}_2\text{SO}_4$  ( $50 \mu\text{L well}^{-1}$ ), and the absorbance was recorded at 450 nm.

## 2.8. Competitive indirect ELISA (ciELISA)

The effect of the different spacer arms of the immunizing haptens on the properties of the antibodies and the heterology of coating antigens on ELISA sensitivity was investigated by competitive indirect ELISA (ciELISA). The checkerboard procedure was used to optimize the coating antigen and the antibody concentrations. For the ELISA, each well of a microtitre plate was coated with  $100 \mu\text{L}$  of the coating antigen at the optimal dilution in carbonate buffer overnight at  $4^\circ\text{C}$ . After washing 5 times with PBST solution, the excess binding sites were blocked with 5% glycine and 5% sucrose in PBS buffer ( $200 \mu\text{L well}^{-1}$ ) for 3 h at  $37^\circ\text{C}$ . After washing 5 times with PBST solution, NPAMTZ standard or sample ( $50 \mu\text{L well}^{-1}$ ) in PBS and polyclonal antibodies diluted in PBST ( $50 \mu\text{L well}^{-1}$ ) were added, incubated for 1 h at  $37^\circ\text{C}$ , and washed 5 times with PBST solution. IgG-HRP diluted 1:10,000 in PBST was added ( $100 \mu\text{L well}^{-1}$ ). After incubation for 1 h at  $37^\circ\text{C}$  and washing 5 times with PBST solution, TMB solution was added to the wells ( $100 \mu\text{L well}^{-1}$ ) and incubated for 15 min. The reaction was stopped by addition of  $2 \text{ mol L}^{-1} \text{H}_2\text{SO}_4$  ( $50 \mu\text{L well}^{-1}$ ), and the absorbance was recorded at

450 nm. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. The sigmoid curves were generated by using OriginPro 7.5 software (OriginLab Corp., Northampton, MA, USA). The limit of detection (LOD) was defined as the concentration of analyte that produced a reduction of 10% of the maximum normalized response ( $IC_{10}$ ). The linear range was defined as the lower and upper limits of quantification, which refers to the  $IC_{20}$ – $IC_{80}$  linear range.

### 2.9. Molecular modeling

The structures of NPAMTZ and the haptens were initially drawn in HyperChem 7.0 software (Hypercube, Inc., USA). The most stable conformations were optimized using Hartree-Fock (HF) with a 6-31G\* basis set in the Gaussian 03 package (revision B.05, Gaussian, Inc., USA). The alignment of haptens and NPAMTZ was completed by SYBYL7.3 program package (Tripos, Inc., USA) on a personal computer.

### 2.10. Samples analysis

For the determination of nitrofurantoin metabolites in animal tissue samples, the first step of the procedure, which was always carried out under mildly acidic conditions, was the release of bound metabolites from the tissue. Next, the derivatisation of metabolites using o-NBA was necessary in order to increase the molecular mass and to improve the sensitivity of detection. The extraction of the nitrophenyl derivatives was carried out using a moderately polar organic solvent such as ethyl acetate, and a clean up step using a non polar solvent such as hexane was used to remove lipids from the sample prior to detection.

Fish, shrimp, chicken and pork samples were purchased from a local market and proved to be free of AMOZ residue by LC-MS/MS, which was completed by the Zhongshan Quality Supervision & Inspection Institute of Agricultural Products of Guangdong Province. The sample preparation was performed according to the method described by Cooper et al. [19] with some modifications. Simply, the samples were homogenized and respectively spiked with different concentrations of AMOZ (in methanol) to final concentrations of 1.0, 5.0 and 20.0  $\mu\text{g kg}^{-1}$ . The spiked sample (1.0 g) was transferred into a glass tube and 4 mL of distilled water, 1.0 mL of HCl (1.0 mol  $\text{L}^{-1}$ ) and 100  $\mu\text{L}$  of *o*-NBA in DMSO (50 mmol  $\text{L}^{-1}$ ) were added successively. The mixture was vortexed for 30 s and incubated overnight at 37  $^{\circ}\text{C}$ . After cooling to room temperature, 0.1 mol  $\text{L}^{-1}$   $\text{K}_2\text{HPO}_4$  (5.0 mL), 1.0 mol/L NaOH (0.4 mL) and ethyl acetate (5.0 mL) were added to the sample, and then shaken vigorously for 30 s. The mixture was centrifuged (3,000  $\times$  g) at room temperature for 10 min. The upper ethyl acetate layer (2.5 mL) was transferred into another glass tube and dried using nitrogen gas at 55  $^{\circ}\text{C}$ . Hexane (0.5 mL) was added to the tube and vortexed thoroughly followed by the addition of 0.5 mL of PBS. After vortexing again, the sample was centrifuged (3,000  $\times$  g) at room temperature for 10 min. The upper hexane layer was removed and the remaining extract was used for analysis.

For ELISA analysis, the extract was diluted three times with the PBS assay buffer. For LC-MS/MS analysis, the extract was filtered with a microporous membrane (0.45  $\mu\text{m}$ ) prior to use. An Agilent ZORBAX SB- $\text{C}_{18}$  (2.1 $\times$ 150 mm, 3.5  $\mu\text{m}$  particle size) column was used. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was acetonitrile. The following gradient profile: 0 min, 22% B; 0–6 min, 22–99% B; 6–9 min, 99%B; 9–9.1

min, 99–22% B; 9.1–15 min, 22% B. The flow rate of the mobile phase was  $0.3 \text{ mL min}^{-1}$  and an aliquot of  $5 \mu\text{L}$  of each sample was injected into the LC system. Analytes were determined by ESI-MS/MS in positive mode. The parameters were as follows: gas temperature,  $350 \text{ }^{\circ}\text{C}$ ; gas flow,  $10 \text{ L min}^{-1}$ ; nebulizer gas, 35 psi; capillary voltage, 4.0 kV. High-purity nitrogen ( $>99.99\%$ ) was served as the nebulizer and collision gas.

### 3. Results and discussion

#### 3.1. Design and synthesis of haptens

Hapten design and synthesis plays a key role in the development of immunoassays for small molecular weight compounds [21]. For nitrofurantoin metabolites, haptens were always designed by derivatising the metabolites with 3-CBA or 4-CBA [6, 7, 9-15]. Although this strategy was able to generate antibodies with high specificity and sensitivity, it's believed that there are some other strategies to further improve the antibody characteristics. Cheng et al. [8] tried to introduce a 6-methoxyhexanoic acid spacer on the 5' position of phenyl ring of NPAOZ and used it as a new immunizing hapten. This hapten possessed the  $-\text{NO}_2$  group and therefore the corresponding antibody showed higher sensitivity and specificity against NPAOZ in ELISA, compared with the antibody generated with CPAMOZ [6-8]. However, the hapten was difficult to synthesize. In this work, four benzaldehyde derivatives (including 4-CBA (hapten 1, containing 1 carbon spacer), 3-CBA (hapten 2, containing 1 carbon spacer), 2-(4-formylphenoxy)acetic acid (hapten 3, containing 2 carbon and 1 oxygen spacer), and 2-(3-formylphenoxy)acetic acid (hapten 4, containing 2 carbon and 1 oxygen spacer)) were attached to AMOZ for immunizing haptens to study the effect of spacer group on production



of specific antibodies against the NPAMOZ. Moreover, another hapten consisting of AMOZ moiety and a simple carboxyl spacer was synthesized as a coating hapten, because previous studies had revealed that using only a partial structure of the target molecule as the coating hapten may be a good strategy to improve assay sensitivity [22,23].

The synthesis of each of the five haptens can be achieved for each using a one step reaction. The synthesized haptens were characterized by ESI-MS and NMR and the data generated is presented, as shown in the previous experimental section 1. This data suggested that the synthesis of all five target haptens (Fig. 1) was successful.

### 3.2. Preparation of hapten-protein conjugates

Four immunizing haptens (Hapten 1 to Hapten 4) were coupled to BSA for immunogens and all the five haptens were coupled to OVA for coating antigens. The synthesized conjugates demonstrated qualitative differences between carrier protein and conjugate in the UV-Vis spectra, suggesting successful hapten conjugation to the carrier protein. The hapten coupling ratios with carrier proteins were examined as 15:1, 15:1, 20:1 and 21:1 for hapten 1-BSA, hapten 2-BSA, hapten 3-BSA and hapten 4-BSA, and 10:1, 11:1, 14:1, 14:1 and 13:1 for hapten 1-OVA, hapten 2-OVA, hapten 3-OVA, hapten 4-OVA and hapten 5-OVA, respectively. The binding ratios of immunogens were suggested to be suitable to evoke a specific immune response [24].

### 3.3. Antisera titer

Eight white New Zealand rabbits were used to prepare polyclonal antisera against four immunogens. All the rabbits' antisera exhibited high levels of polyclonal antibodies; the titer

values after the 3<sup>rd</sup> injection were 0.84-1.32 at 1: 60,000 dilutions in homologous indirect ELISA. Antiserum R215, R217, R220 and R221 that showed higher titers in indirect ELISA and better sensitivity in ciELISA were used in subsequent studies.

### 3.4. Characterization of polyclonal antibodies

To study the effects of the immunizing haptens and heterologous coating on assay sensitivity, ciELISA based on different antiserum (R215, R217, R220 and R221, respectively) and different coating (hapten 1-OVA, hapten 2-OVA, hapten 3-OVA, hapten 4-OVA and hapten 5-OVA, respectively) were developed. The IC<sub>50</sub> values for NPAMAZ are displayed in Table 1.

#### 3.4.1. Effect of immunizing haptens

As shown in Table 1, the averaged IC<sub>50</sub> values for NPAMAZ based on antiserum R215 (refer to hapten 1, 1C spacer), R217 (refer to hapten 2, 1C spacer), R220 (refer to hapten 3, 2C and 10 spacer) and R221 (refer to hapten 3, 2C and 10 spacer) in homologous ciELISA were 55.6, 51.3, 32.1 and 30.1  $\mu\text{g L}^{-1}$  respectively. The location of the carboxy spacer on the phenyl ring of immunizing haptens did not significantly influence the sensitivity of antibody in ciELISA. However, antibodies R220 and R221 showed improved sensitivity for NPAMAZ in ciELISA in comparison with antibodies R215 and R217, which indicated that the length of spacer on the phenyl ring of immunizing haptens did significantly influence the sensitivity of antibody in ciELISA. Those antibodies raised to haptens 3 and 4 incorporating the phenoxy group displayed almost a 2 fold increase in sensitivity.

It is well known that the three dimensional structure is important for the antibody recognition of low molecular weight haptenic compounds [25]. The introduction of a linker or spacer arm between the hapten and carrier protein may cause perturbations in the three-dimensional conformation of the hapten [26]. Therefore, the lowest energy conformations were calculated for the free haptens and NPAMMZ in this study. Based on these stable conformations, each immunizing hapten was matched with NPAMMZ and the results are shown in Fig. 2. Clearly, the part of the AMMZ structure of hapten 3 and hapten 4 were well matched with that of NPAMMZ (Fig. 2, C and D). However, this matching was not obtained in the alignment of hapten 1 and hapten 2 with NPAMMZ. An obvious conformational change was observed at the structure of morpholine ring for both hapten 1 and hapten 2 (Fig. 2, A and B). The introduction of a carboxyl group on the phenyl ring might influence the electronic distribution of phenyl ring and then further influence the conformation of AMMZ structure. While introducing a spacer between the carboxyl group and the phenyl ring, this influence may decrease. Therefore, the introduction of a certain length of spacer between the carboxyl group and the phenyl ring is important for producing an antibody with improved sensitivity for NPAMMZ.

#### 3.4.2. *Effect of heterologous assay format*

It's generally accepted that heterology usually results in weaker recognition of antibody to the ELISA plate-coating antigen compared to the recognition of the target analyte. Thus, lower analyte concentrations can compete with these reagents, which results in improved assay sensitivity [27,28]. However, the design of an optimal heterologous coating hapten is not so easy. As shown in Table 1, for each antibody, the heterologous coating can improve

the assay sensitivity, but not all the improvements are noteworthy. For examples, for antibody R215, when using hapten 2-OVA, hapten 3-OVA and hapten 4-OVA, the assay sensitivity was not improved significantly. This might be due to the low degree of hapten heterology. However, the assay sensitivity for all four antibodies was greatly improved when using hapten 5-OVA as the coating antigen. A 2 to 3 fold increase in sensitivity was obtained for all four antibodies when hapten 5-OVA was used as the coating antigen. Hapten 5 exhibited the AMOZ moiety, which was a partial structure of the target molecule (NPAMOZ). The result of this study demonstrated a previous conclusion that using only a partial structure of the target molecule as the coating hapten may be a good strategy to improve assay sensitivity [22,23].

### 3.5. Optimization of ELISA conditions

With the aim to improve the ciELISA performance the influence of several factors (pH, ionic strength and Tween-20 concentration) ciELISA were examined. For each condition, the standard curves for NPAMOZ were established ( $n=3$ ) and the maximum absorbance value ( $B_0$ ) as well as the concentration of analyte that produces a 50% decrease in  $B_0$  ( $IC_{50}$ ) were obtained. The  $B_0/IC_{50}$  ratio was used to estimate the effect of a certain factor on the ciELISA performance, the higher ratio indicating the higher sensitivity response to the condition being tested [29,30]. As shown in Fig. 3A, both the absorbance value and the sensitivity increased first and then decreased with pH values ranging from 5.0 to 9.0. The highest  $B_0/IC_{50}$  value was obtained when the pH value was around 7.0. Therefore, it was chosen as the optimal pH of the buffer. For ionic strength, with the increasing concentration of salt, the absorbance value increased gradually and reached a peak at  $40 \text{ mmol L}^{-1}$ , and then

decreased (Fig. 3B), which indicated that a high salt concentration might inhibit the antibody-antigen interaction. The salt concentration at  $20 \text{ mmol L}^{-1}$  appeared to be the optimal condition for the competition buffer since the highest value of  $B_0/IC_{50}$  was obtained. Tween-20 is a nonionic surfactant commonly used in immunoassay protocols to reduce non-specific interactions [30]. In this work, the effect of Tween-20 concentration on ELISA performance was also studied. As shown in Fig. 3C, low content of Tween-20 in the assay buffer was advantageous for the antibody-antigen interaction and the competition of analyte. The presence of 0.01% Tween-20 in the assay buffer provided the best assay sensitivity.

As a result of the immunoassay evaluation described above we can conclude that some factors allowed the improvement of the ELISA performance. The optimal assay buffer was  $20 \text{ mmol L}^{-1}$  of PBS at pH value of 7.0, containing 0.01% Tween-20. Under the optimum conditions, the dose-response curve for NPAMOZ in assay buffer was developed and shown in Fig. 4. The  $IC_{50}$  value for NPAMOZ is  $2.1 \mu\text{g L}^{-1}$ , the limit of detection (LOD) is  $0.1 \mu\text{g L}^{-1}$  and the linear range is  $0.3\text{--}35.1 \mu\text{g L}^{-1}$ .

Until now, only one other publication described and reported the development of an ELISA for AMOZ [15]. In that research, AMOZ was derivatised with 3-CBA and used as an immunizing hapten. The  $IC_{50}$  value for NPAMOZ ranged from  $1.59\text{--}72.3 \mu\text{g L}^{-1}$  in the homologous ciELISA was based on different monoclonal antibodies. Our results indicated that by using the new immunizing hapten (hapten 4) and the heterologous coating system, the assay sensitivity for NPAMOZ was improved. In our lab, the research is now focusing on the screening of hybridomas that secrete monoclonal antibodies against NPAMOZ (based on

the novel immunogen, hapten 4-BSA). It is envisaged that the production of monoclonal antibodies with higher sensitivity to NPAMAZ can be achieved.

### 3.6. Antibody specificity

Nitrofurans and their metabolites, the nitrophenyl derivatives of metabolites, and several other veterinary drugs commonly used in fishery were used to test the specificity of antibody R221. Table 2 presents the results as percentage cross-reactivity with respect to the target molecule, NPAMAZ. The data showed that antibody R221 had a significant cross-reactivity (34.4%) with its parent drug furaltadone and little cross-reactivity (2.3%) with AMOZ. This indicated that the antibody recognized the bridge at the –N–N position. The reduced cross-reactivity with furaltadone should be due to the replacement of the phenyl ring by furan ring. The loss of nitrophenyl bridge resulted in a great reduction of cross-reactivity for AMOZ. The antibody exhibited negligible binding with other nitrofurans metabolites and their nitrophenyl derivatives. Among them, NPAOZ shared a large common structure to NPAMAZ (other than the morpholine ring derivative). This result indicated that the morpholine ring derivative should be a characteristic moiety for antibody recognition. From the three dimensional structure of NPAMAZ, the morpholine ring derivative formed a big cleft with the moiety of 3-aminooxazolidin-2-one, which should be for antibody binding. A similar result was also obtained in the research by Pimpitak et al. [15]. The antibody also showed no cross-reactivity to various veterinary drugs or the derivatising agent o-NBA. It is well known that nitrofurans are metabolized rapidly *in vivo*, and as a consequence the significant cross-reactivity with furaltadone is unlikely to affect the determined results in practice. The negligible assay response to o-NBA (<0.02%) eliminates the need for separation

of this reagent from the sample homogenate after derivatisation when employing published extraction procedures [12].

### 3.7. Analysis of samples

Fish, shrimp, chicken and pork samples with a confirmed absence of AMOZ were spiked at three different concentrations (1.0, 5.0 and 20.0  $\mu\text{g kg}^{-1}$ ) with AMOZ and analyzed by the developed ciELISA. The results were validated with the standard LC-MS/MS. For ciELISA analysis, the sample extracts were first determined without further pretreatment. Slightly lower recoveries (average of 74.7% and ranging from 68.5% to 81.2%, Table 3) were obtained. In contrast, good recoveries (average of 94.9% and ranging from 79.5% to 107.2%, Table 3) were obtained for the standard LC-MS/MS analysis. The comparative lower recovery for the ciELISA analysis maybe due to the trace amounts of solvent (hexane) left in the sample extract. On dilution of the sample extracts with PBS the recoveries were tested again. The results indicated that good recoveries (average of 91.1% and ranging from 81.5% to 102.3%, Table 3) were obtained when three dilutions with PBS were performed. Following the correction of the data by a dilution factor of three, the LOD and linear range of the developed ciELISA for AMOZ in fish and shrimp samples were 0.3  $\mu\text{g kg}^{-1}$  and 0.9 to 105.3  $\mu\text{g kg}^{-1}$ , respectively. The LOD of this assay can satisfy the MRPLs (1  $\mu\text{g kg}^{-1}$ ) for AMOZ set by the EU.

The ciELISA was then applied to determine AMOZ residue in market samples. Twenty samples (including five fish samples (#1 to #5), five shrimp samples (# 6 to # 10), five chicken samples (#11 to #15) and five pork samples (#16 to #20)) were collected from the markets nearby the South China Agricultural University. One shrimp sample (#7) was found

AMAZ positive ( $3.4 \mu\text{g kg}^{-1}$  by ciELISA). The following HPLC-MS/MS analysis gave AMAZ value of  $4.2 \mu\text{g kg}^{-1}$ . No false positive and negative results were obtained in the screening test. It indicated that the assay is ideally suited as a screening method for AMAZ residue prior to chromatographic analysis.

#### 4. Conclusions

Two novel immunizing haptens (derivatised 3-amino-5-morpholinomethyl-2-oxazolidinone with 2-(4-formylphenoxy)acetic acid or 2-(3-formylphenoxy)acetic acid) were designed and coupled to bovine serum albumin as immunogens to raise antibodies against the nitrophenyl derivative of furaltadone metabolite 3-amino-5-morpholinomethyl-2-oxazolidinone (NPAMAZ). New Zealand rabbits were immunized with the immunogens and polyclonal antibodies specific to NPAMAZ were obtained. The results indicated antibodies produced against the novel immunizing haptens showed higher sensitivity in competitive indirect enzyme-linked immunosorbent assay (ciELISA) compared to antibodies produced against the traditional immunizing haptens (derivatised 3-amino-5-morpholinomethyl-2-oxazolidinone with 3-carboxybenzaldehyde or 4-carboxybenzaldehyde). Molecular modeling was used to optimize the lowest energy conformations of NPAMAZ and the immunizing haptens. The results indicated that the two novel haptens had better alignments with NPAMAZ than the two traditional haptens. A novel heterologous coating hapten was also synthesized derivatising 3-amino-5-morpholinomethyl-2-oxazolidinone with 2-oxoacetic acid. Marked improvement on assay sensitivity was observed when using a heterologous ELISA assay format, which suggested that the strategy of using only a partial structure of the target



molecule as the coating hapten can improve the assay sensitivity. The ciELISA based on the optimal antibody and coating antigen showed an  $IC_{50}$  of  $6.3 \mu\text{g L}^{-1}$ , the limit of detection (LOD) of  $0.3 \mu\text{g L}^{-1}$  and the linear range of  $0.9\text{--}105.3 \mu\text{g L}^{-1}$  for NPAMOZ in animal samples. Thus the LOD of this assay can satisfy the MRPLs ( $1 \mu\text{g kg}^{-1}$ ) for AMOZ in food of animal origin set by the EU. These results indicated that the proposed ELISA, with high sensitivity and specificity, as well as good reproducibility and accuracy, is ideally suited as a monitoring method for AMOZ residues at trace levels. Subsequently, effort will be made on the screening of monoclonal antibodies against NPAMOZ from mice immunized with the novel immunogen (hapten 4-BSA).

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**Table 1**

IC<sub>50</sub> values ( $\mu\text{g L}^{-1}$ ) for NPAMTZ to indicate the effects of immunogens and heterologous coating on the ciELISA sensitivity

Immunogen	Antiserum	Coating antigen				
		Hapten 1- OVA	Hapten 2- OVA	Hapten 3- OVA	Hapten 4- OVA	Hapten 5- OVA
Hapten 1- BSA	R215	55.6 $\pm$ 4.4	45.4 $\pm$ 4.1	35.2 $\pm$ 2.8	31.7 $\pm$ 3.6	17.1 $\pm$ 1.5
Hapten 2- BSA	R217	35.7 $\pm$ 3.2	51.3 $\pm$ 6.0	28.3 $\pm$ 2.7	33.4 $\pm$ 3.9	14.3 $\pm$ 2.3
Hapten 3- BSA	R220	29.0 $\pm$ 2.6	24.2 $\pm$ 2.5	32.1 $\pm$ 2.5	20.6 $\pm$ 1.8	9.9 $\pm$ 1.1
Hapten 4- BSA	R221	21.9 $\pm$ 1.9	26.8 $\pm$ 3.1	21.7 $\pm$ 2.3	30.1 $\pm$ 2.5	7.6 $\pm$ 0.9

**Table 2**

Cross-reactivity of R221 antibody with NPAMTZ and other structurally related compounds or commonly used veterinary drugs

Competitor	IC <sub>50</sub> (µg L <sup>-1</sup> )	CR <sup>a</sup> (%)
NPAMTZ	2.1±0.14	100
NPAOT, NPSEM, NPAHD	>10 000	<0.02
AMTZ	89.7±9.4	2.3
AOZ, SEM, AHD	>10 000	<0.02
Furaltadone	6.1±4.6	34.4
Nitrofurantoin, nitrofurazone, furazolidone	>10 000	<0.02
o-NBA	>10 000	<0.02
Ciprofloxacin	>10 000	<0.02
Enrofloxacin	>10 000	<0.02
Malachite-green	>10 000	<0.02

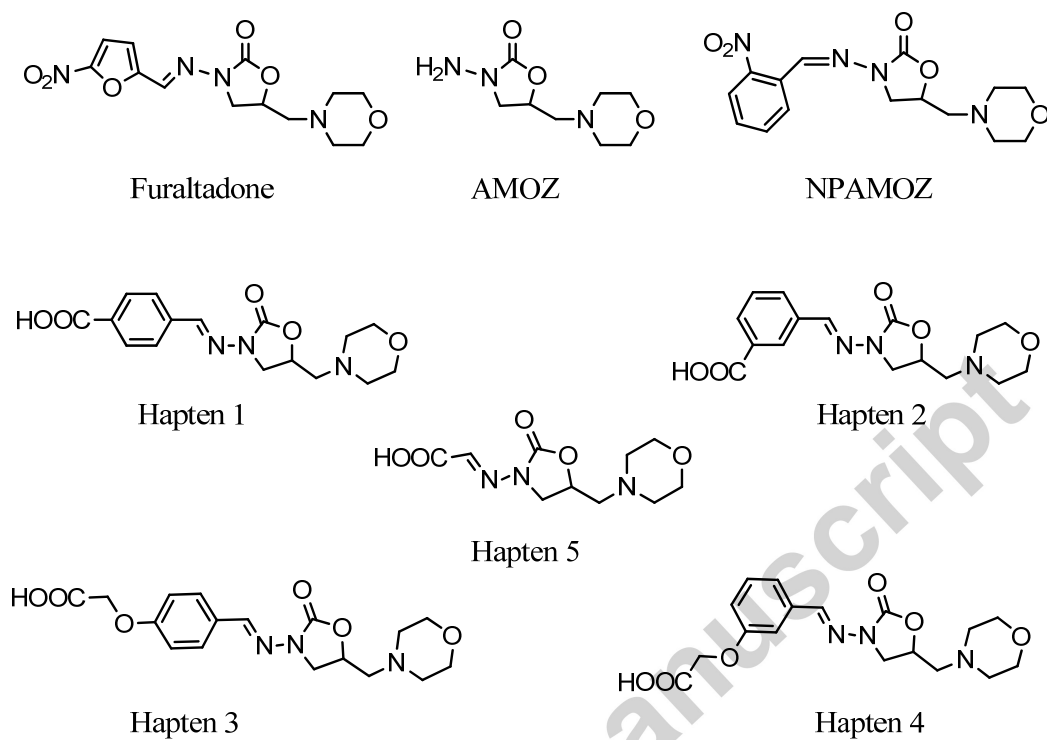
<sup>a</sup> CR = cross-reactivity. Percentage of CR was calculated according to the following equation:

$$[\text{IC}_{50}(\text{NPAMTZ}, \mu\text{g L}^{-1}) / \text{IC}_{50}(\text{cross-reactant}, \mu\text{g L}^{-1})] \times 100.$$

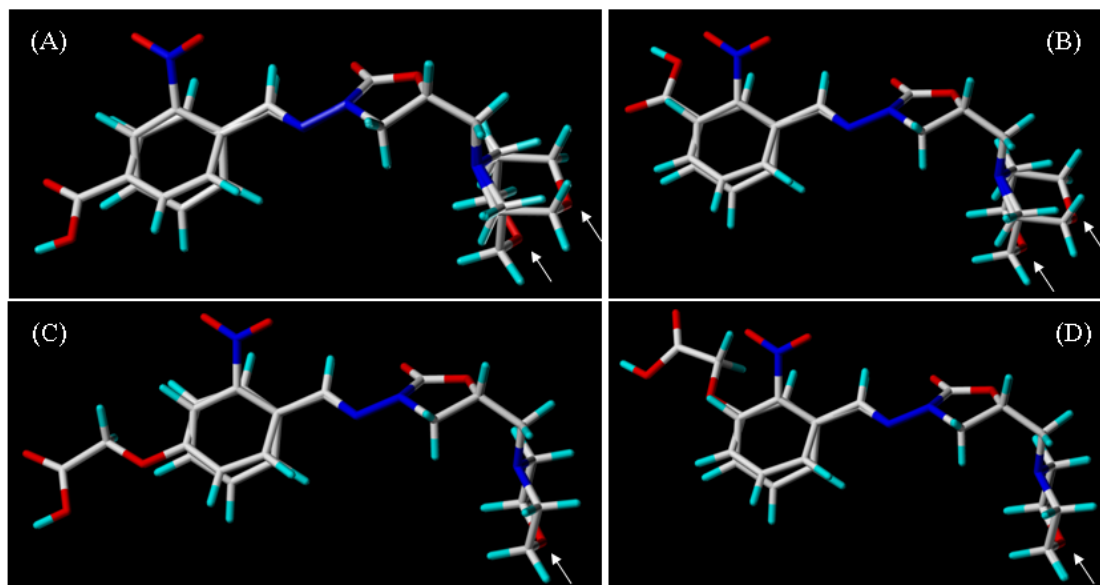
**Table 3**

Recoveries of AMOZ from spiked animal tissue samples by ciELISA and LC-MS/MS (n=3)

Sample	Spiked ( $\mu\text{g kg}^{-1}$ )	ciELISA				LC-MS/MS	
		No dilution of extract		3 times dilution of extract		Recovery (%)	CV (%)
		Recovery	CV (%)	Recovery	CV (%)		
		(%)		(%)			
Fish	1.0	74.0	11.3	82.4	12.8	97.4	5.6
	5.0	69.5	13.9	88.0	10.3	89.6	10.9
	20.0	71.2	14.6	95.6	15.1	105.6	7.6.
Shrimp	1.0	73.4	11.5	81.5	12.7	107.2	6.3
	5.0	70.1	16.2	97.3	11.6	82.7	4.4
	20.0	77.6	13.9	85.7	8.9	95.4	8.0
Chicken	1.0	80.3	9.7	91.6	11.6	79.5	11.3
	5.0	74.2	15.0	102.3	10.7	103.2	8.6
	20.0	79.1	11.3	95.5	9.8	95.4	9.0
Pork	1.0	68.5	15.2	91.6	15.6	97.3	7.9
	5.0	81.2	9.8	87.9	13.4	98.8	10.1
	20.0	77.5	14.1	94.3	10.9	86.8	6.5

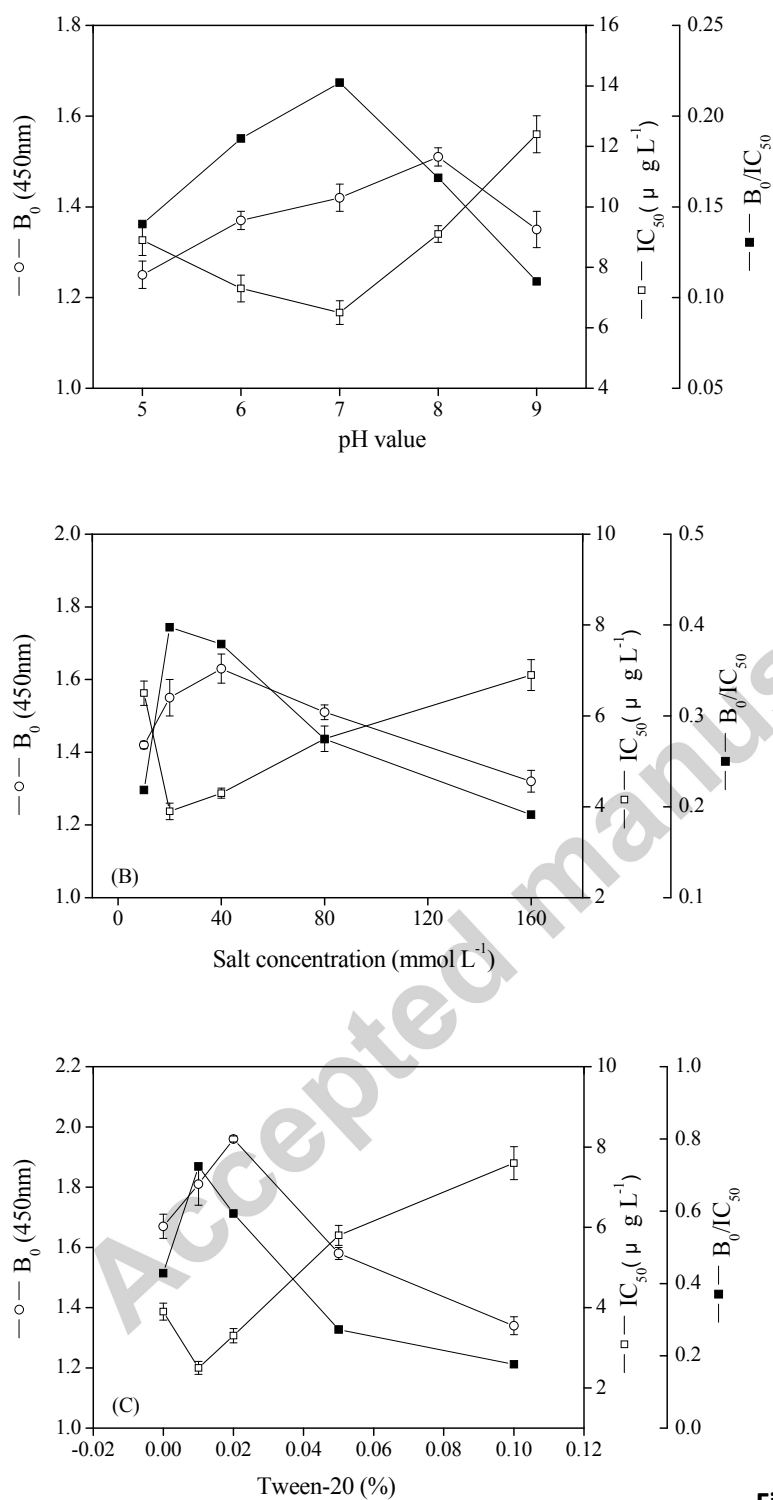


**Fig. 1.** Chemical structure of furaltadone, its metabolite AMOZ, the detecting target NPAMOZ and the haptens (hapten 1 to hapten 4 were used for immunizing haptens and hapten 5 was used for coating haptens).



**Fig. 2.** The overlap of NPAMTZ and four immunizing haptens based on the lowest energy conformations. (A) NPAMTZ and hapten 1; (B) NPAMTZ and hapten 2; (3) NPAMTZ and hapten 3; (4) NPAMTZ and hapten 4. The arrows point at the morpholine ring, the conformation of which was changed after introducing of different spacers.

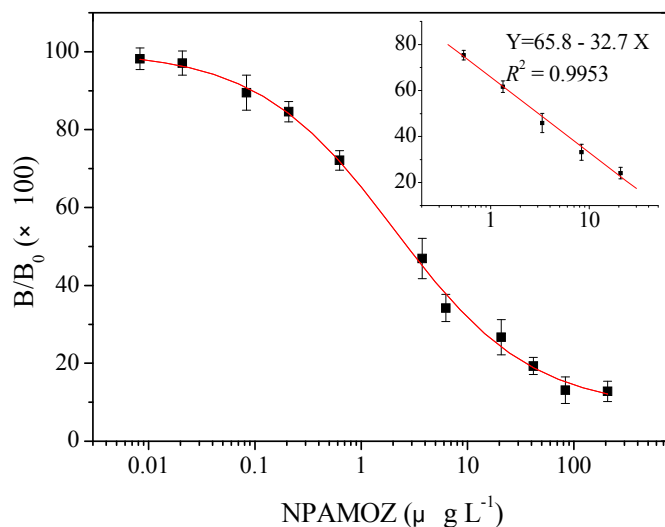




**Fig. 3.** Influence of pH (A),

ionic strength (B), and concentration of Tween-20 (C) on the ciELISA of NPAMAZ. Each point

represents the average of three replicates and the standard deviation of the mean.



**Fig. 4.** Dose-response curve and calibration curves in the linear range (insert) for NPAMOZ in assay buffer. The microtiter plate was coated with hapten 5-OVA at  $10 \mu\text{g L}^{-1}$ ; antibody (R221) dilution = 1: 20000; competitive time = 1.0 h; IgG-HRP dilution = 1: 10000. Each point represents the average of three replicates and the standard deviation of the mean.

## Highlights

- > Several novel haptens against furaltadone metabolite AMOZ were synthesized.
- > Polyclonal antibodies against the novel haptens were generated.
- > Improved sensitivity was observed for the antibodies in ELISA.
- > Molecular modeling of haptens structure was used to explain the results.
- > Application of ELISA in real sample indicated good reproducibility and accuracy.
- > The assay is ideally suited as a monitoring method for AMOZ residues at trace level.